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Uptake and Distribution of ^{14}C-Tepa in Boar Spermatozoa After in Vitro Treatment

ABSTRACT

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The sperm-rich fraction of boar semen was treated in vitro for 10 minutes with an equal volume of 1 percent solution of ^{14}C-labeled tepa. On average, 0.8 percent was taken up in the spermatozoa; 69 percent was associated with the heads and 31 percent with the tails and acrosomes. Radioautography of the intact, treated spermatozoa revealed that the labeled tepa was attached but that the binding site could not be determined. Fractionation of the sperm heads yielded a digested protein fraction that contained 65 percent of the initial uptake. Further processing of the crude deoxyribonucleic acid (DNA) fraction gave a good recovery of DNA containing less than 1 percent protein, but radioassay of the isolated DNA from treated sperm showed no trace of the carbon-14 label.

KEYWORDS: Boar spermatozoa, fractionation, radioautography, sperm DNA, ^{14}C-tepa.

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UPTAKE AND DISTRIBUTION OF ^{14}C-TEPA IN BOAR SPERMATOZOA AFTER IN VITRO TREATMENT

by Jerry B. Stokes, Alexej B. Borkovec, and Charles W. Woods[1/]

The induction of dominant lethal mutations in boar spermatozoa treated in vitro with tepa (tris(1-aziridinyl)phosphine oxide) was demonstrated by Pursel et al. (7).[2/] Tepa-treated spermatozoa exhibited normal motility and fertilizing ability in artificial insemination tests, but the zygotes did not develop to the blastocyst stage. Similar activity of tepa in spermatozoa of honey bees (Apis mellifera L.) had been reported previously by Taber and Borkovec (10). Apparently in both studies the aziridinyl alkylating agent tepa reacted directly with the sperm cells, and this reaction produced specific changes in the genetic components of the cells that were later manifested as dominant lethal mutations. Since the generalized hypothesis of the action mechanism of alkylating agents (4), and more specifically of aziridines (9), implicates the deoxyribonucleic acids (DNA) of the cells as the target molecules, we used boar semen as a model system for studying the chemical changes induced by ^{14}C-labeled tepa in sperm.

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2/ Underlined numbers in parentheses refer to Literature Cited at the end of this report.

MATERIALS AND METHODS

TREATMENT OF SEMEN

Tepa (500 mg) containing ^{14}C-labeled tepa (6.6 μCi, sp. act. 20 mCi/mmol) in 50 ml of Beltsville L1 extender (8) was added to 50 ml of the sperm-rich fraction of semen (5-10 X 10^8 sperm/ml) from mature boars 2 hours after collection. Ten minutes after the tepa was added, the sample was centrifuged at 300 g for 15 minutes, and the seminal plasma was decanted. The sperm fraction was washed with 50 ml of Beltsville L1 extender, and the centrifugation was repeated. After three additional washings and centrifugation with 50 ml of standard saline citrate (SSC, 0.15 M sodium citrate), the radioactivity of the washings was equivalent to the background. The radioactivity of each washing was determined by liquid scintillation counting in Aquasol. The percentage uptake of labeled tepa in spermatozoa was determined by a wet combustion technique (3) and liquid scintillation counting. The centrifuged pellet of spermatozoa was weighed and a one-tenth aliquot was transferred to a small piece of 6-mm glass tubing. The sample was oxidized, and the labeled carbon dioxide was trapped in 6 ml of ethanolamine:methyl Cellosolve (1:1). A 3-ml aliquot was combined with 15 ml of Aquasol and assayed by liquid scintillation counting.

FRACTIONATION OF SPERM

Hotta and Bassel (5) isolated boar sperm DNA by a procedure that included an enzymatic digestion at 60° C that was not adaptable to our needs. Korban and Sharobaiko (6) extracted DNA from boar spermatozoa by a procedure similar to that of Borenfreund et al. (2) to isolate DNA from mammalian spermatozoa. Our method is an expansion of that of Borenfreund et al. (2), and it provided suitable conditions for measuring the label distribution. Thus spermatozoa obtained after the extender washings were resuspended and centrifuged three times with SSC and once with 0.05 M Tris HCl, pH 8.0. Then they were blended in a Waring blendor at low speed for 5 minutes to cleave the tails. The heads were collected after 15 minutes' centrifugation at 300 g through a bed of 0.6 M sucrose, 0.05 M Tris HCl, pH 8.0. The uptake of labeled tepa by the heads was determined by the same procedure except that we used a one-tenth aliquot of the centrifuged pellet of sperm heads. For a determination of the uptake of tepa by the sperm tails and acrosomes, a one-tenth aliquot of the sucrose supernatant was added directly to the combustion apparatus.

To remove lipoproteins, we treated the sperm heads with two volumes of 1 percent Triton X-100 (v/v) in 0.05 M Tris HCl, pH 8.0, with slow stirring at 5° C overnight. After sedimentation at 300 g, the sperm heads were treated at 40° for 1 hour with 0.01 M sodium deoxycholate in 0.05 M Tris HCl, pH 8.0, and centrifugation was repeated. The disulfide linkages of the sperm heads were cleaved by an overnight treatment with 2 percent (v/v) 2-mercaptoethanol in SSC at 5° with stirring.

Trypsin (200 mg, Sigma #T-8128) was added to the suspension, the pH was adjusted to 7.8 with 1 N sodium hydroxide, and the mixture was incubated at 37° for 3-4 hours. The viscous solution was centrifuged at 38,000 g for 1 hour, and the pellets were suspended in SSC containing 2 percent (v/v) 2-mercaptoethanol and digested as before with 100 mg of trypsin. The supernatants were pooled, 7 percent (w/v) of 4-amino-salicylic acid and 2 percent (w/v) of sodium dodecyl sulfate were added, the pH was adjusted to 7.5, and the solution was stirred for 1 hour at ambient temperature.

The digested protein was extracted by slow stirring with liquid phenol (saturated with SSC) for 30 minutes, the mixture was centrifuged for 20 minutes at 300 g, and the aqueous phase was decanted. This extraction was repeated four times. A 5-ml aliquot was added directly to the combustion apparatus to measure the label that was released by the digestion procedure. The aqueous layer was washed three times with 100 ml of diethyl ether to remove excess phenol followed by two washings with 50 ml of chloroform:isoamyl alcohol (3:1). Sodium dodecyl sulfate (5 percent in 45 percent ethyl alcohol (EtOH), 0.1 vol.) was added, and the mixture was stirred at room temperature for 3 hours. Solid sodium chloride (NaCl) was added to give a salt concentration of 1 M, and the mixture was stirred for 1 hour. Then it was placed in an ultracentrifuge for 1 hour at 15,000 g, and the DNA was precipitated from the supernatant by adding two volumes of cold 95 percent EtOH. The DNA fibers were loosely wrapped on a glass rod and redissolved in 0.01 M Tris HCl, pH 8.0, containing urea, NaCl, magnesium dichloride ($MgCl_2$), and 2-mercaptoethanol at final concentrations of 5 M, 4 M, 0.001 M, and 0.01 M, respectively. After mild shaking for 3 hours at ambient temperature, the solution was centrifuged for 1 hour at 15,000 g. The supernatant was concentrated by ultrafiltration (Amicon PM10 membrane filter) and eluted from an Agarose column (BioRad A50) with 0.01 M Tris HCl, pH 8.0, containing urea, NaCl, $MgCl_2$, and 2-mercaptoethanol in the proportions mentioned previously. The pooled fractions of DNA were dialyzed against 0.01 M Tris HCl, pH 7.5, and aliquots were taken for standard DNA, ribonucleic acid, and protein determinations. Incorporation of label into the isolated DNA was measured by liquid scintillation counting after the entire sample was oxidized in the combustion apparatus. The yield of dry weight DNA was 1.3-1.4 mg per milliliter of semen (72-78 percent based on the estimation method of Anand et al. (1)).

RADIOAUTOGRAPHY

The labeled spermatozoa were mixed with warm molten 2 percent agar and allowed to solidify. The solidified agar was diced into 2- to 3-mm cubes and then transferred to glass vials containing 3 percent glutaraldehyde in 0.05 M phosphate buffer, pH 6.8, at 22° C for chemical fixation. After 1.5 hours'

fixation, the agar blocks were washed in six changes of the buffer over a period of 1 hour. They were postfixed in 2 percent osmium tetroxide for 2 hours. Then they were dehydrated in an acetone series and infiltrated with a low viscosity embedding medium. Ultrathin sections were prepared on nickel grids, and the loop technique was used to layer the Ilford L4 emulsion. The grids were covered, attached to glass slides with tape, transferred to light-tight boxes, and kept at 4° for 4-6 weeks. Microdol X was used as the developer after the emulsion was stabilized for 30 minutes in 70 percent ethanol vapor. The grids were then examined in the electron microscope. The DNA isolated from the tepa-treated spermatozoa was prepared for radioautography by placing 1.3-1.4 mg on a microscope slide and then drying it in an evacuated desiccator overnight. The slide was exposed to Kodak SB-5 X-ray film in a light-tight desiccated container at -20° for 4 to 8 months.

RESULTS

UPTAKE AND DISTRIBUTION OF LABELED TEPA IN SPERM

After a 10-minute treatment of 50 ml of semen with labeled tepa (6.6 μCi, 1.465×10^7 dpm), 0.8 percent (1.17×10^5 dpm) of the label (four replicates), on an average, was associated with the sperm cells. Radioautography of individual spermatozoa revealed the presence of radiocarbon, but no specific information could be obtained about its location in the cell. When sperm heads were separated from tails and acrosomes by sucrose gradient centrifugation, about 69 percent (8.07×10^4 dpm) of the label (two replicates) was found in the heads, and about 31 percent (2.63×10^4 dpm) remained in the tails and acrosomes fraction. This distribution pattern does not indicate any preferential binding of the mutagen to the cell nucleus.

INCORPORATION OF LABELED TEPA INTO DNA

Further fractionation of the head fraction of the treated spermatozoa, with cleavage of disulfide linkages and trypsin digestion, yielded a digested protein fraction that was evaluated for radioactivity. Approximately 65 percent (7.59×10^4 dpm) of the label (two replicates) was associated with this fraction. Further processing of the crude DNA fraction yielded pure DNA containing less than 1 percent protein. No radioactivity was detected by wet combustion and liquid scintillation counting. Radioautography failed to show any label attached to the isolated DNA.

DISCUSSION

Although tepa is an effective mutagen of boar sperm (7), our study does not support the hypothesis that direct alkylation of the sperm DNA is the mode of action. Evidently the low level of tepa (0.8 percent) that was incorporated into the spermatozoa was associated primarily with the sperm proteins and not with the DNA. The extent of interaction of tepa with DNA or with DNA-containing cellular materials has not been studied previously, but Trams et al. (11) reported that a related aziridine, 2,4,6-tris(1-aziridinyl)-s-triazine (TEM), was incorporated into the DNA fraction of regenerating liver at a level of 1 mol of TEM per 1 mol of DNA. Similar levels of incorporating tepa into boar sperm DNA would have been easily detected in our study. However, we cannot say that some tepa did not react with the sperm DNA initially and that during fractionation and purification the labeled substituents were not cleaved off and separated from the purified DNA. The probability of such dealkylation appears low, but it cannot be reliably determined unless a reaction product of tepa and DNA is isolated and chemically identified.

LITERATURE CITED

(1) Anand, A. S., W. G. Hoekstra, and N. L. First. 1967. Effect of aging boar spermatozoa on cellular loss of DNA. J. Anim. Sci. 26: 171-173.

(2) Borenfreund, E., E. Fitt, and A. Bendich. 1961. Isolation and properties of deoxyribonucleic acid from mammalian sperm. Nature [London] 191: 1375-1378.

(3) Chang, S. C., A. B. Borkovec, and C. W. Woods. 1966. Fate of tepa uniformly labeled with C^{14} in male house flies. J. Econ. Ent. 59: 937-944.

(4) Freese, E. 1971. Molecular mechanism of mutations. In Hollaender, A., ed., Chemical Mutagenesis, pp. 1-56. Plenum Press, New York.

(5) Hotta, Y., and A. Bassel. 1965. Molecular size and circularity of DNA in cells of mammals and higher plants. Natl. Acad. Sci. Proc. 53: 356-362.

(6) Korban, N. V., and V. I. Sharobaiko. 1973. Extraction of DNA from boar sperm. In Lebedev, M. M., and V. G. Bekhtina, eds., Biokhim. Metody Issled. Spermy Selsk. Khoz. Zhivotn., pp. 36-41. [CA 84: 40464v (1976).] [In Russian.]

(7) Pursel, V. G., L. A. Johnson, and A. B. Borkovec. 1975. Effects of in vitro treatment of boar spermatozoa with tepa on the fertilization and development of pig eggs. J. Reprod. Fert. 45: 549-552.

(8) Pursel, V. G., L. A. Johnson, and L. L. Schulman. 1973. Fertilizing capacity of boar semen stored at 15° C. J. Anim. Sci. 37: 532-535.

(9) Stokes, J. B. 1979. Interaction of aziridines with cellular components. U.S. Dept. Agr. ARM-NE-3, 38 pp.

(10) Taber, S., and A. B. Borkovec. 1969. Chemical sterilization of honey bee spermatozoa in vitro. Nature [London] 224: 1217-1218.

(11) Trams, E. G., M. V. Nadkarni, and P. K. Smith. 1961. On the mechanism of action of the alkylating agents. I. Interaction of alkylating agent with nucleic acids. Cancer Res. 21: 560-566.

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